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INVESTIGATION INTO THE BIOLOGY OF STEMPHYLIUM BOTRYOSUM, A POTENTIALLY NEW PATHOGEN IN LENTIL PRODUCTION IN SASKATCHEWAN

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Investigation into the biology of *Stemphylium botryosum*, a potentially new pathogen in lentil production in Saskatchewan

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(a) Abstract/Summary

Stemphylium botryosum sporulates poorly under laboratory conditions compared to other lentil pathogens. Evaluating different incubation periods and conditions revealed that incubation of at least 25 days resulted in significantly higher spore yields than shorter incubation periods. Intervals of light or darkness during that period had no effect on sporulation. Best sporulation was obtained on a V8 - potato dextrose medium. The age of the fungal culture from which spores were harvested had no influence on germination. Inoculation of lentil plants with different concentrations of spores of *S. botryosum* resulted in significantly higher disease severity on plants inoculated at a higher concentration. Lentil plants appear to be more susceptible the more mature they are at the time of infection.

Exposing spore suspensions on glass slides as well as inoculated plants to a range of wetness periods at different temperatures revealed a significant interaction between temperature and wetness periods. Longer wetness periods were required at lower temperatures for spore germination and for achieving higher disease severity. Highest germination rates and highest disease severity on CDC Milestone were observed at 25 to 30°C. However, even at 5°C approximately 25% of conidia had germinated after 12 h of incubation in wet conditions, and low levels of disease developed at temperatures of 10°C. The effect of intermittent dry periods depended upon the length of that period, the temperature, the duration of the initial wet period and the period of wetness the spores were exposed to in total. Expanding dry periods beyond 12 h with a total wetness duration of 8 h resulted in a significant decrease in spore germination. After an initial wetness period longer than 4 hours spores appeared to be somewhat more susceptible to drying. When air humidity was maintained at 80% during the dry period, the fungus appeared to be less sensitive to dry periods at 30°C compared to 25 or 20°C. This may be due to the fact that its development is faster at this temperature thus dry-sensitive stages in the development are passed through quicker. Increasing initial wetness periods resulted in higher disease severity, suggesting that more spores were successful at invading the longer they were exposed to free water on the leaf surface. This effect was more significant than the duration of the dry period.

Experiments on yield loss under controlled conditions showed that the resistant variety Eston and the susceptible variety CDC Milestone varied in their responses to infection at different stages of their development. Yield and quality reduction in Eston only occurred when plants were infected very early. In contrast, CDC Milestone also responded with yield and quality loss when infected at later stages of plant development. Inoculating both varieties at mid and again at late podding, and maintaining them under moist conditions resulted in seed infection levels of 82% for CDC Milestone and 71% for Eston, which is significantly higher than what has been observed in field samples, probably due to the fact that infection in the field seems to occur shortly before maturation of lentil plants.

Screening of different isolates of *S. botryosum* showed that they did not react differently on different varieties, suggesting that there are no races in the population. Evaluating different lentil varieties for resistance to stemphylium blight showed that Barimasur 4 and Eston were the most resistant cultivars with significantly lower disease levels compared to all other lentil cultivars. CDC Glamis was the most susceptible variety with significantly higher levels of stemphylium

blight compared to all other lentil cultivars tested. CDC Blaze, CDC Milestone, CDC Redcap, CDC Robin and CDC Vantage had comparable, intermediate levels of resistance.

Among the fungicides tested Lance showed highest efficacy in controlling *S. botryosum* on lentil. It showed highest inhibition of fungal growth in plate tests and best control of the pathogen on inoculated lentil plants. Quadris also inhibited fungal development whereas Headline and Bravo treated plants had similar levels of disease as the unsprayed control plants.

(b) Executive Summary

Stemphylium botryosum causes leaf blight on lentil that can result in large-scale defoliation of plants. The fungus has been a serious pathogen in Bangladesh for many years where yield losses of 62 % and more have been experienced due to the disease. In recent years, *Stemphylium* has been observed increasingly in lentil fields in Saskatchewan and on seed tested for infection with *A. lentis* and other common lentil pathogens (R. Morrall, pers. com.). *S. botryosum* is a poorly researched fungus and no information is available on the pathogen on lentil in a semi-arid climate. This research project was initiated to investigate the effect of plant age, the optimum of various environmental factors for *S. botryosum*, such as temperature and wet periods, the effect of stemphylium blight on biomass and seed production of lentil, the level of seed infection, and the efficacy of fungicides against the pathogen.

Stemphylium botryosum sporulates poorly under laboratory conditions compared to other lentil pathogens, which was unexpected when initiating this project. As a consequence, several experiments had to be conducted to identify the best method for producing spores that were essential for all inoculation experiments. Evaluating different incubation periods and conditions revealed that incubation of at least 25 days resulted in significantly higher spore yields than shorter incubation periods. Intervals of light or darkness during that period had no effect on sporulation. In previous experiments V8 juice medium had been identified as the best medium for sporulation. Screening of various supplements showed that a combination of V8 and potato dextrose agar resulted in a dramatic increase in sporulation compared to straight V8 medium. Nevertheless, spore numbers of *S. botryosum* harvested from a single Petri plate are still significantly lower than what is harvested from plates inoculated with any other pathogen in our laboratory. Because large amounts of inoculum are required for large scale screening in the breeding program, a protocol was developed to use mycelium rather than spores for inoculations.

Because of this shortage of spores, we conducted initial experiments on spore germination on glass slides. The age of the fungal culture from which spores were harvested had no influence on germination, nor did spores germinate differently under light or in the dark. Temperature had a significant effect on germination and was significantly higher at 25 to 30°C. This suggests that the fungus has a similar temperature optimum than *Colletotrichum truncatum*, the pathogen that causes anthracnose on lentil. However, spores did germinate at temperatures as low as 5°C and reached 30 to 40% after 20 h of incubation, and low levels of disease developed at temperatures of 10°C. This observation was very important as rain events in the Prairies even in summer are usually associated with lower temperatures. This suggests that infection takes place under relatively cool conditions.

The effect of intermittent dry periods depended upon the length of that period, the temperature, the duration of the initial wet period and the period of wetness the spores are exposed to in total. Interrupting germinating spores after 2 h of wet conditions by exposing them to dry periods of various lengths resulted in a significant reduction in germination. Spores appeared to be sensitive to dry periods longer than 12 h. Expanding dry periods beyond 12 h with a total wetness duration of 8 hours results in a significant decrease in spore germination. After an initial wetness period longer than 4 hours spores appeared to be somewhat more susceptible to drying than earlier suggesting that they may reach a critical point in their development that is more sensitive to drying than other stages of development. When air humidity was maintained at 80% during the dry period, the fungus appeared to be less sensitive to dry periods at 30°C compared to 25 or 20°C. This may be due to the fact that its development is faster at this temperature thus dry-sensitive stages in the development are passed through quicker. Increasing initial wetness periods resulted in higher disease severity, suggesting that more spores were successful at invading the longer they were exposed to free water on the leaf surface. This effect was more significant than the duration of the dry period.

Periods of wet and dry conditions are characteristic for the situation in the filed during the growing season in the prairies. Thunder storms often create periods of leaf wetness of various lengths, after which spores are exposed to dry periods before another thunder storm or a dew period allows them to continue germination and infection. Different fungi have different levels of sensitivity to dry period as an adaptation to the environment they inhabit.

Inoculation of lentil plants with different concentrations of spores of *S. botryosum* resulted in significantly higher disease severity on plants inoculated at a higher concentration. Lentil plants appeared to be more susceptible to the disease the closer they got to maturity. However, the impact of the disease on biomass production, seed yield and quality was dependent on the variety and on the level of resistance a genotype posses. The more susceptible variety CDC Milestone reacted with a significant decrease in biomass and seed yield when plants were inoculated later than 2 weeks after seeding. Eston, as a more resistant variety, only responded with yield and biomass loss when inoculated 2 weeks after seeding, but not later. Evaluating different lentil varieties for resistance to stemphylium blight showed that that Barimasur 4 and Eston were the most resistant cultivars with significantly lower disease levels compared to all other lentil cultivars. CDC Glamis was the most susceptible variety with significantly higher levels of stemphylium blight compared to all other lentil cultivars tested. CDC Blaze, CDC Milestone, CDC Redcap, CDC Robin and CDC Vantage had comparable, intermediate levels of resistance. Barimasur 4 has been used in the breeding program as a source of resistance to stemphylium blight. It would be interesting to investigate whether Eston possesses the same resistance gene(s) or different genes, in which case pyramiding of genes may be of interest. Screening of 10 different isolates of *S. botryosum* showed that they did not react differently on different varieties, suggesting that there are no races in the population.

Plate tests evaluating fungal growth on media amended with fungicides at different concentrations, and plant test under controlled conditions were conducted to evaluate the efficacy of fungicides registered in lentil against *S. botryosum*. Among the fungicides tested Lance showed highest efficacy in controlling *S. botryosum* on lentil. It showed highest inhibition of fungal growth in plate tests and best control of the pathogen on inoculated lentil plants. Quadris also inhibited fungal development whereas Headline and Bravo sprayed plants had

similar levels of disease as the unsprayed control plants. These results suggest that producers have effective fungicides available to control the pathogen.

(c) Technical Report

Background:

Stemphylium botryosum causes leaf blight on lentil that can result in large-scale defoliation of plants. The fungus has been a serious pathogen in Bangladesh for many years where yield losses of 62 % and more have been experienced due to the disease. In recent years, *Stemphylium* has been observed increasingly in lentil fields in Saskatchewan and on seed tested for infection with *A. lentis* and other common lentil pathogens (R. Morrall, pers. com.). *S. botryosum* is a poorly researched fungus and no information is available on the pathogen on lentil in a semi-arid climate. It is not known whether this fungus has the same climatic optima as *A. lentis* and *C. truncatum*, whether it competes with these pathogens on the host plant lentil, whether it is favored on new, ascochyta blight and anthracnose resistant cultivars because of lack of competition, whether it has the potential to cause severe economic losses, or whether it is controlled by the same disease management strategies implemented for ascochyta blight and anthracnose control.

Project Objectives:

The objectives of this project were to investigate the effect of plant age, the optimum of various environmental factors (e.g. temperature and wet periods) for the development of *S. botryosum*, the effect of stemphylium blight on biomass and seed production of lentil and the level of seed transmission. Furthermore, field isolates were evaluated for variability in virulence on lentil, and the effect of fungicides commonly used in lentil on the fungus was tested. Results from this project enabled us to develop efficient screening method to select for resistance to the disease. It also provides essential information to assess the severity of threat this disease may pose to lentil production in Saskatchewan.

1. Identification of a suitable medium and conditions for high sporulation of *Stemphylium botryosum*

Initial attempts in growing *S. botryosum* on standard media to produce spores for inoculation showed that in contrast to other lentil pathogens this fungus did not sporulate profusely and that spores were difficult to harvest. Therefore experiments were conducted to identify a more suitable medium for sporulation and to optimize incubation conditions.

1.1 Identification of a medium for sporulation

Material and Methods:

Isolate SB-19 was inoculated onto the following media with 4 replicate plates per medium, and incubated for 11 days in light at 27°C:

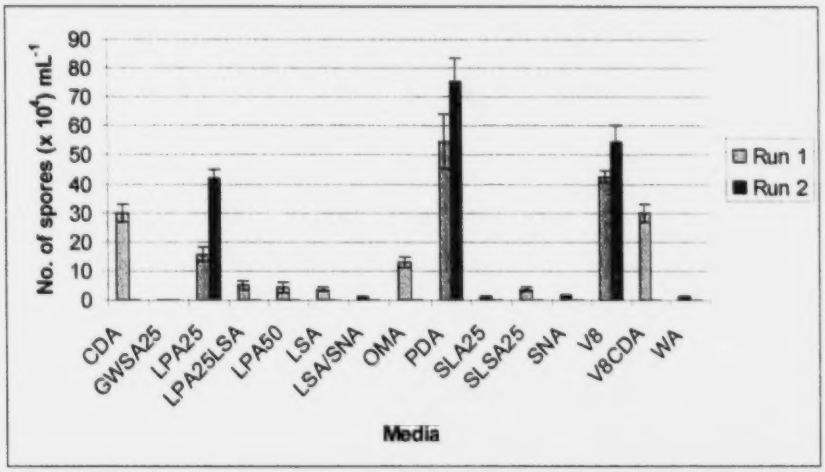
1) **PDA:** 25% Strength Potato Dextrose Agar (PDA) (6.75 g PDA, 8.44 g agar, 750 mL distilled water),
2) **OMA:** 50% Oatmeal Agar (30 g powdered oatmeal, 6.25 g agar, 1 L distilled water),
3) **WA:** Water Agar (11.25 g agar, 750 mL tap water),
4) **LSA:** Lentil Seed Agar (100 g lentil seed, 10 g plain agar, 1.25 L de-ionized waster),
5) **SNA:** Salt Nutrient Agar (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4$-7 H_2O, 0.5 g KCl, 0.2 g Glucose, 0.2 g Sucrose, 20 g Agar, 1L de-ionized H_2O),
6) **LSA/SNA:** 100% Lentil Seed + 100% Salt Nutrient Agar (100 g lentil seed, 1g KH_2PO_4, 1g KNO_3, 0.5 g $MgSO_4$-7 H_2O, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 20 g agar, 1 L di-ionized H_2O),
7) **LPA25:** 25% Lentil Powder Agar (15 g lentil flour, 6.25 g agar, 1 L di-ionized water),
8) **LPA50:** 50% Lentil Powder Agar (30 g lentil flour, 6.25 g agar, 1 L di-ionized water),
9) **LPA25LSA:** 25% Lentil Powder + 100% Lentil Seed Agar (100 g lentil seed, 15 g lentil flour, 6.25 g agar, 1 L di-ionized water),
10) **SLA25:** 25% Ground Lentil Stem Agar (15 g ground lentil stems and leaves, 6.25 g agar, 1 L di-ionized water),
11) **SLSA25:** 25% Ground Lentil Stem, Leaf and Seed Agar (15 g ground lentil stems, leaves and seeds, 6.25 g agar, 1 L di-ionized water),
12) **GWSA25:** 25% Ground Wheat Straw Agar (15 g ground wheat straw, 6.25 g agar, 1 L di-ionized water),
13) **V8:** V8 Juice Agar (1.13 g $CaCO_3$, 11.3 g agar, 112.5 mL V8 juice, 637.5 mL di-ionized water),
14) **CDA:** Carrot Dextrose Agar (24 g carrot, 12 g dextrose, 17 g agar, 1 lit distilled water),
15) **V8CDA:** Carrot Dextrose Agar/ V8 juice (24 g carrot, 12 g dextrose, 17 g agar, 150 ml V8 juice, 850 mL distilled water).

After the first run of comparison, media with highest sporulation were re-tested to confirm results.
Cultures were washed with 5mL di-ionized water adding one drop of Tween 20 and using a paint brush. The suspension in the Petri dishes was not filtered to achieve maximum spore concentrations. Spore concentration was counted with a haemocytometer.
Data was tested for homogeneity of variance and normal distribution, and was transformed wherever necessary. Analysis of variances was conducted using either the GLM procedure or the MIXED procedure in SAS. Means were either separated by LSD or Tukey statistics.
Comparisons between treatments using contrasts were made wherever appropriate.

Results:

Comparison of sporulation on various media revealed that among media tested with isolate SB-19 highest sporulation was obtained on V8 juice media (V8) media with 43 to 55 x 10^4 spores mL^{-1} followed by lentil flower (LPA) at a concentration of 50% with 16 to 42 x 10^4 spores mL^{-1} and 25% potato dextrose agar (PDA) with 53 to 76 x 10^4 spores mL^{-1} (Fig. 1.1).

Fig. 1.1. Comparison of sporulation of *Stemphylium botryosum* isolate SB-19 on 15 different media incubated for 11 days after incubation under light at a temperature of 27°C. Y-bars are standard errors of the means.



1.2 Identification of optimum incubation conditions for sporulation

Further experiments were conducted in an attempt to improve spore production by *S. botryosum*.

Material and Methods:

A randomized complete block design with 4 replicates was used to study the effect of different incubation conditions on sporulation of *S. botryosum*. Isolate SB-19 was inoculated onto V8 juice medium and incubated at 27°C. Cultures were incubated for the following incubation period:

1) 7days light;
2) 11days light;
3) 7 days light/2 days dark/2 days light (After 7 days light 2 mL of sterile di-ionized water was added, the cultures was gently brushed with a paint brush and mycelium and spores re-distributed on the plate.);
4) 15 days light;
5) 7 days light/2 days dark/8 days light (After 7 days light 2 mL of sterile di-ionized water was added, the cultures was gently brushed with a paint brush and mycelium and spores re-distributed on the plate.);
6) 7 days light/2 days dark/14 days light (After 7 days light 2 mL of sterile di-ionized water was added, the cultures was gently brushed with a paint brush and mycelium and spores re-distributed on the plate.),

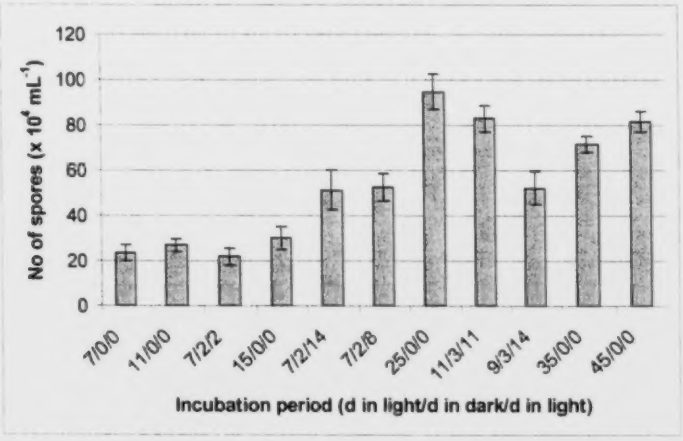
7) 9 days light/3 days dark/14 days light (After 9 days light 2 mL of sterile di-ionized water was added, the cultures was gently brushed with a paint brush and mycelium and spores re-distributed on the plate.),
8) 11 days light/3 days dark/11 days light (Cultures were kept in the dark for 3 days, after adding water and re disturbing the spore and mycelia),
9) 25 days light, 35 days light, 45 days light.

Spores were then washed from the plates using 5 mL of sterile di-ionized water adding one drop Tween 20 and using a paint brush. Spore concentration was counted with a haemocytometer. The experiment was conducted twice.
Data was tested for homogeneity of variance and normal distribution, and was transformed wherever necessary. Analysis of variances was conducted using either the GLM procedure or the MIXED procedure in SAS. Means were either separated by LSD or Tukey statistics.
Comparisons between treatments using contrasts were made wherever appropriate.

Results:

Exposing culture of *S. botryosum* to varying incubation conditions with regard to incubation time and light exposure revealed that increasing the period of incubation resulted in higher sporulation (Fig.1.2). Contrasting all incubation treatments with continuous light versus those with dark periods revealed no significant differences in sporulation. This was also true when contrasting a 25 day incubation period in light with an incubation period of 11 days in the light, 3 days in dark and 11 days in light.
Significantly lower sporulation was obtained when incubation period was restricted to 15 days or less. Increasing the incubation period from 25 to 45 days had no significant effect on sporulation suggesting that a 25 day incubation period in the light may be the most efficient approach for producing spores.

Fig. 1.2. Spore concentrations obtained from Petri plates inoculated with *Stemphylium botryosum* and incubated for different incubation periods and with different light regimes. Y-bars are standard errors of the means.



1.3 Testing the effect of various supplements to V8 juice medium on sporulation of *S. botryosum*

Additional experiments were conducted to investigate whether the addition of specific sugars, starch, CO_3Ca or glycerol induced higher sporulation on V8 medium.

Materials and Methods:

Isolate SB-19 was grown on V8 juice medium with several supplements in a randomized complete block design with 4 replicates and incubated for 11 days in light, 3 days dark followed by 11 days light. After the first incubation period of 11 days in the light, 2 mL of sterile di-ionized water was added, the cultures was gently brushed with a paint brush to re-distribute mycelium and spores on the plate prior to further incubation in the dark and light. The following supplements were added to V8 juice medium:

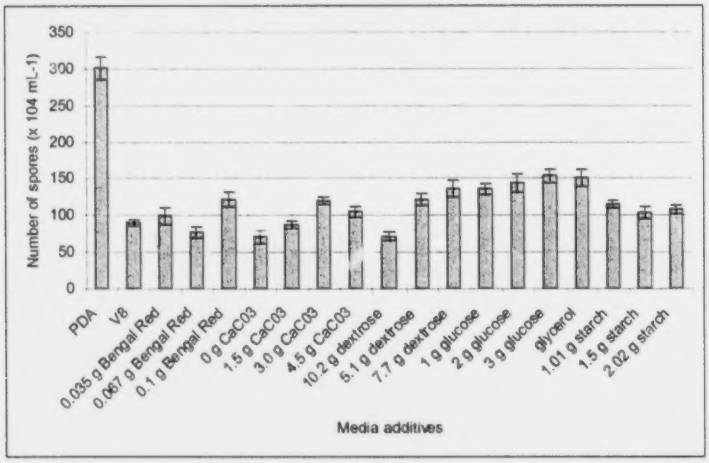
1) V8 Juice Agar (V8) (1.13g $CaCO_3$, 11.3g agar, 112.5mL V8 juice, 637.5 mL di-ionized water),
2) V8 with 0.035 g rose bengal,
3) V8 with 0.06, 0.1, g rose bengal,
4) V8/Potato Dextrose Agar (PDA: 1.5g $CaCO_3$, 15g agar, 15 g potato dextrose agar, 150 mL V8 juice, 850 mL di-ionized water),
5) V8 with 5.1 g dextrose,
6) V8 with 7.7 g dextrose,
7) V8 with 10.2 g dextrose,
8) V8 with 1.01 g corn starch,
9) V8 with 1.5 g corn starch,
10) V8 with 2.02 g corn starch,
11) V8 with 1.5 g CO_3Ca,
12) V8 with 3 g CO_3Ca,
13) V8 with 4.5 g CO_3 Ca,
14) V8 with 1 g glucose,
15) V8 with 2 g glucose,
16) V8 with 3 g glucose,
17) V8 with glycerol (1.13g $CaCO_3$, 11.3g agar, 9.2 mL glycerol ($C_3H_3O_3$)112.5mL V8 juice, 637.5 mL di-ionized water).

Spores were washed from the plate with 5 mL of sterile di-ionized water adding one drop Tween 20 and using a paint brush. Spore concentrations were counted with a haemocytometer. The experiment was conducted twice.
Data was tested for homogeneity of variance and normal distribution, and was transformed wherever necessary. Analysis of variances was conducted using either the GLM procedure or the MIXED procedure in SAS. Means were either separated by LSD or Tukey statistics. Comparisons between treatments using contrasts were made wherever appropriate.

Results:

Addition of PDA to the V8 juice medium dramatically increased sporulation by approximately 300 % compared to straight V8 juice medium (Fig.1.3). A significant, though much smaller increase in sporulation compared to V8 was also achieved by adding 7.7 g dextrose, glucose and glycerol suggesting that *S. botryosum* requires a nutrient-rich medium for sporulation.

Fig. 1.3. Spore concentrations obtained from *Stemphylium botryosum* grown on a V8 juice medium with different additives. Y-bars are standard errors of the means.



General Discussion:

Spore production of *S. botryosum* was highest on a medium consisting of V8 juice and potato dextrose where we harvested a maximum of 1.5×10^7 spores per Petri plate. This is 1/10 of the average amount of spores we usually harvest from other lentil pathogens like *Ascochyta lentis* or *Colletotrichum truncatum*, thus requiring us to prepare 10 times more plates for spore production than we require for screening with these other pathogens. In addition, experience with *S. botryosum* in the course of these epidemiological studies has shown that sporulation is variable from plate to plate and over time. While producing spores of *S. botryosum* is still feasible with small-scale experiments, large-scale screening of germplasm for the identification of resistance is not, and alternative screening methods have to be found.

2. Preliminary spore germination studies

Two preliminary experiments were conducted to investigate the effect of culture age and incubation in light or in darkness of spore germination for subsequent spore germination studies.

2.1 The effect of culture age on spore germination

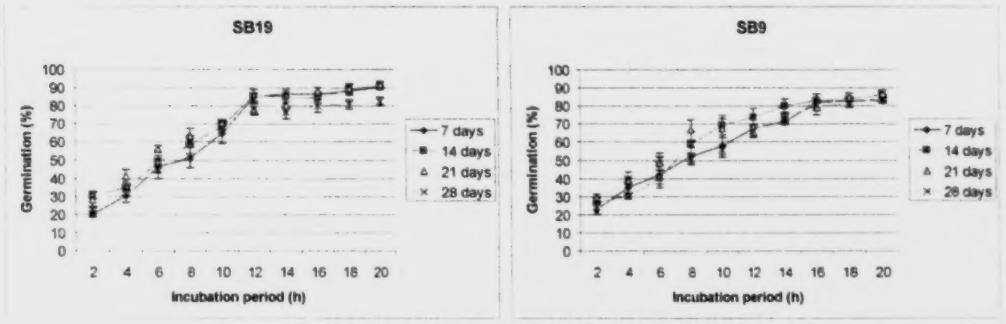
Materials and Methods:

Isolates SB-19 and SB-9 were grown on 4 replicate Petri plates with a V8/PDA medium for periods of 7, 14, 21 and 28 days. Spores were harvested from the plates by flooding the plates with 5mL of sterile water and a drop of Tween 20, and then gently dislodging the spores with a soft brush. All spore suspensions were processed simultaneously to avoid any possibility of bias. Spore concentration was adjusted to 2×10^4 spores mL^{-1}. An aliquot of 100µl of spore suspension for each plate was spread on well slides and incubated at high humidity in a Petri dish with moistened filter paper at 25°C. For each slide 100 spores were counted within several fields of view selected from left to right across each slide. Spores were counted at two hour intervals for 20 hours, to determine the effect of culture age on spore germination. Spores were considered to have germinated when the germ tube is at least half the width of the spore. The number of germinated spores was expressed as a percentage of the total number of spores counted.
Data was tested for homogeneity of variance and normal distribution, and was transformed wherever necessary. Analysis of variances was conducted using either the GLM procedure or the MIXED procedure in SAS. Means were either separated by LSD or Tukey statistics. Comparisons between treatments using contrasts were made wherever appropriate.

Results:

Harvesting spores from cultures of 7, 14, 21 and 28 days did not affect the sporulation rate over a period of 20 h of both isolates (Fig.2.1). After 2 hours of incubation spores of all cultures had a germination rate between 20 to 30 % which increased to 80 to 90 % over the course of 20 h incubation. Analysis of the percentage germination after 20 h revealed no significant difference between spores originating from cultures of different ages. This suggests that for further experimentation spores of cultures of any age can be used without affecting the germination rate.

Fig 2.1. Percent of germination of conidiospores of *Stemphylium botryosum* isolates SB-9 and SB-19 harvested from cultures after 7, 14, 21 and 28 days and incubated on glass slides over a period of 20h. (Y-bars are standard errors of the means)



2.2 The effect of light on germination

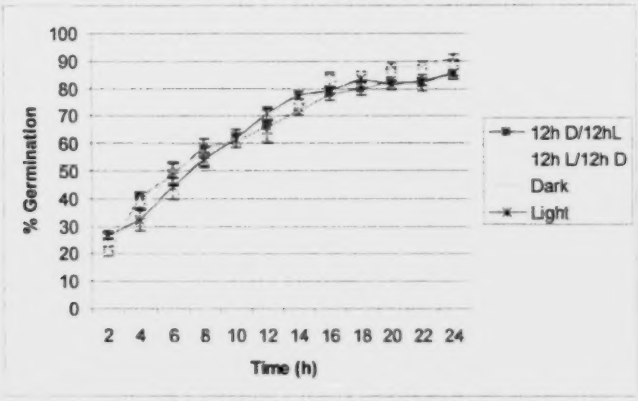
Materials and Methods:

Isolates SB-19 and SB-9 were grown on V8/PDA medium for 7 days, spores were harvested and adjusted to a concentration of $5x10^4$ spores mL^{-1} as described above. Aliquots of 100µl of spore suspension were spread on well slides and incubated at high humidity in a Petri plates with moistened filter paper. Petri plates were incubated at 25°C in either continuous light, continuous dark, or 12 h light and 12 h dark to study the effect of light on germination. Percentage germination was first assessed at 2 hours after the inoculum was placed on the glass well slide, and then every hour until 24 h after inoculation of the well slides.

Results:

Light had no effect on germination over a period of 20h (Fig. 2.3).

Fig. 2.3. Percent of germination of conidiospores of *Stemphylium botryosum* isolate SB-19 incubated in 24h dark, 24h light, 12h dark and 12h light, and 12h light and 12h dark on glass slides over a period of 20h. Y-bars are standard errors of the means.



General Discussion:

Preliminary experiments showed that spore germination was not influenced by the age of the fungal culture at the time of harvest or by the light regime during incubation.

3. Preliminary plant inoculation studies

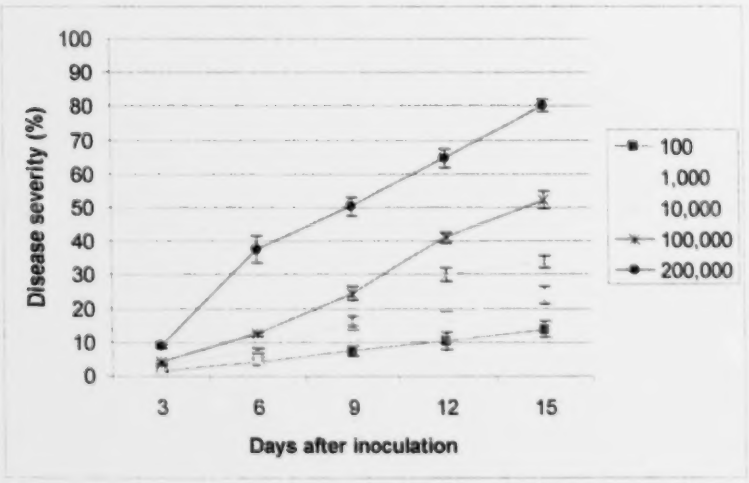
Preliminary studies were conducted to determine the optimum spore concentration for plant inoculation and to develop a new rating scale that was taking account of specific symptoms observed on lentil plants infected with *S. botryosum*.

3.1 The effect of different spore concentrations on disease severity

Materials and Methods:
Isolate SB-19 was grown on V8/PDA and incubated at 27°C for 25 days (11 days light/ 3 days dark/11 days light). The susceptible cultivar CDC Milestone was planted in 6 replications at a density of 2 seeds per 4 inch pot and inoculated at 2 weeks after seeding with the following spore concentration: $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$ and $2x10^5$ spores mL^{-1}. The spore suspensions were applied with an air brush and a carbon dioxide applicator at 5 ml of spore suspension per plant, which was equivalent to run off. Following inoculation, plants were placed in a mist chamber for 48 hr. All treatments were kept in mist bench during the ratings. Disease severity was rated 3 days after inoculation using the Horsfall-Barrett scale (0-11). The ratings were repeated 5 times every 4 days.
Data was tested for homogeneity of variance and normal distribution, and was transformed wherever necessary. Analysis of variances was conducted using either the GLM procedure or the MIXED procedure in SAS. Means were either separated by LSD or Tukey statistics.
Comparisons between treatments using contrasts were made wherever appropriate.

Results:
Increasing spore concentration in steps of one logarithm increased the severity of stemphylium blight significantly (Fig. 3.1). Inoculating plants with a suspension of $1x10^5$ spores mL^{-1} resulted in disease severity levels of over 50% after 15 days. Doubling the spore concentration to $2x10^5$ spores mL^{-1} increased severity by approximately 25%. When evaluating the impact of a disease on plant productivity, yield quantity and quality, high disease levels are desirable to estimate the maximum damage of the disease. Therefore, inoculations would be conducted with the higher concentrations (e.g. $1x10^5$ spores mL^{-1}, $2x10^5$ spores mL^{-1}). For breeding purposes, intermediate disease severity levels are desirable, in particular where disease resistance is considered to be a quantitative trait, in order to identify subtle differences between genotypes. In this case somewhat lower spore concentrations are used (e.g. $2x10^4$ spores mL^{-1}, $1x10^5$ spores mL^{-1}).

Fig. 3.1. Disease severity on plants inoculated with spore suspensions of various concentrations (spores mL^{-1}) of *Stemphylium botryosum*. Y-bars are standard errors of the means.



3.2. Development of a new rating scale

Difficulties were encountered in using the Horsefall-Barratt scale to evaluate disease severity of lentil plants infected by *S. botryosum*. This was primarily due to the fact that the major disease symptom is leaf drop in addition to the development of necrotic area on lentil tissue. The Horsefall-Barratt scale assesses necrotic area of plant tissue and initially attempts were made to assess leaf drop with this scale rather than necrotic area. As genetic studies of resistance in lentil to this disease are conducted parallel to this project we wanted to develop a scale that could potentially be used for quantitative genetic analysis. The Horsefall-Barratt scale is a logarithmic scale and therefore not suitable for this kind of analysis.

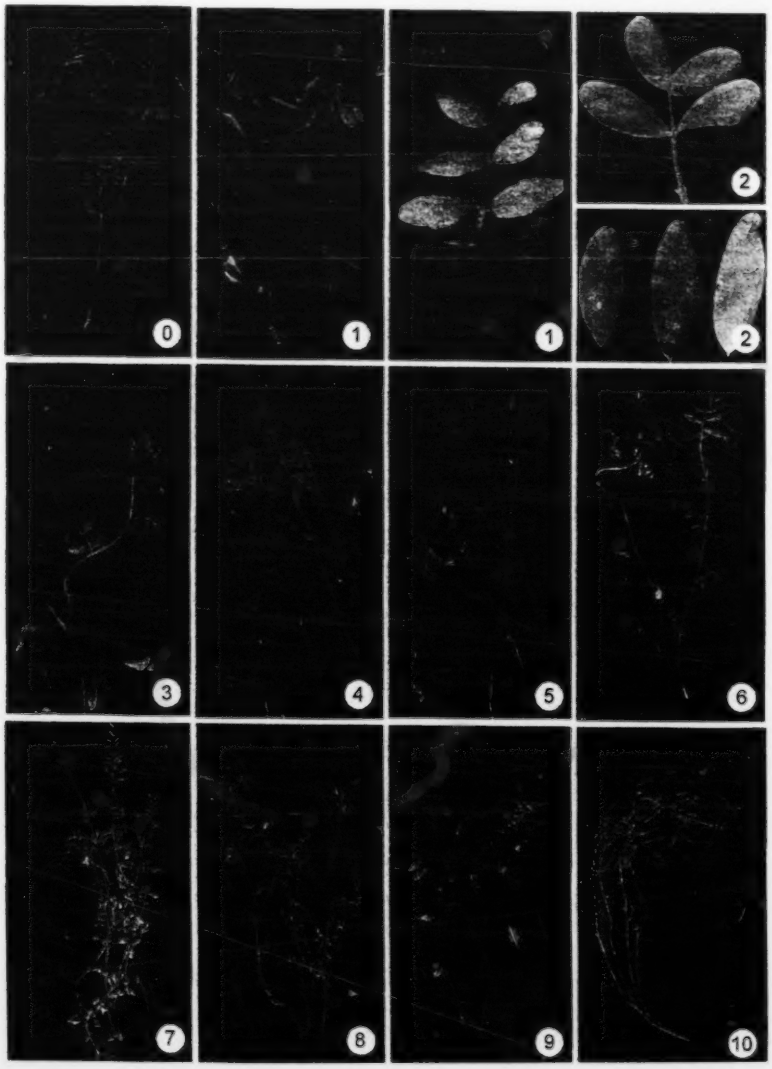
Materials and Methods:

Plants from the experiment described under 3.1 were used to develop a semi-quantitative scale.

Results:

In all experiments conducted so far the Horsefall-Barratt scale has been used to evaluate disease. Though overall a convenient scale, for stemphylium blight several draw-backs of the scale as described in Materials and Methods lead us to develop a new semi-quantitative scale (Fig. 3.2). This scale takes into account lesion development as well as leaf drop and is relatively simple thus allowing large numbers of plants to be evaluated in relatively short periods of time. In the framework of this project, the scale will primarily be used for breeding-related experiments whereas we will continue to use the Horsefall-Barratt scale for epidemiology experiments in order to maintain consistency with experiments that have already been conducted using that scale.

Fig 3.2. Semi-quantitative rating scale developed for stemphylium blight on lentil. 0: healthy plants; 1: few tiny lesions; 2: a few chlorotic lesions; 3: expanding lesions on leaves, onset of leaf drop; 4: $1/5^{th}$ of nodes affected by lesions and leaf drop; 5: $2/5^{th}$ of nodes affected; 6: $3/5^{th}$ of nodes affected; 7: $4/5^{th}$ of nodes affected; 8: all leaves dried up; 9: all leaves dried up but stem green; 10: plant completely dead.



4. Effects of plant age on the development of stemphylium blight

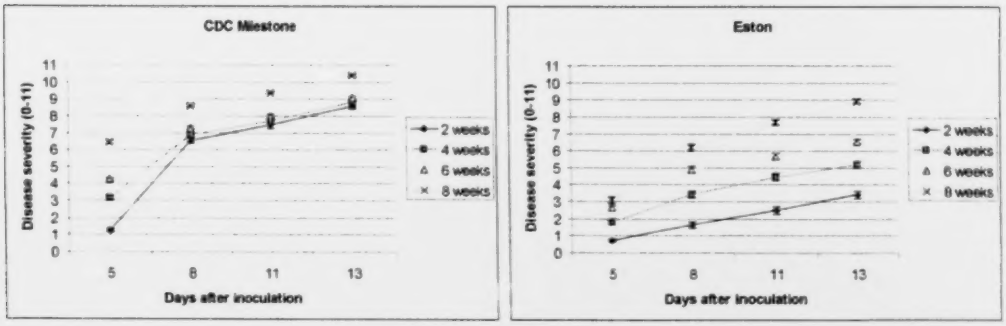
Materials and Methods:

Isolate SB-19 was grown on V8/PDA and incubated at 27 ^{O}C for 25 days (11 days light/ 3 days dark/11 days light). Harvested spore concentration was adjusted to 2 x 10^5 spores mL^{-1}. Plants of Eston and CDC Milestone were grown for 2, 4, and 6 and 8 weeks, were inoculated with the spore suspension and incubated for 48 h under high humidity. Plants were rated 4 times starting 5 days after inoculation at 3 day interval using the Horsefall-Barratt scale, and the area under the progress curve was calculated.
Data was tested for homogeneity of variance and normal distribution, and was transformed wherever necessary. Analysis of variances was conducted using either the GLM procedure or the MIXED procedure in SAS. Means were either separated by LSD or Tukey statistics.
Comparisons between treatments using contrasts were made wherever appropriate.

Results:

On both varieties, plant age had a significant effect on the severity of stemphylium blight (Fig. 4.1). With increasing age lentil plants developed more disease during 13 days of incubation. On plants of the more susceptible variety CDC Milestone, the rate of increase in disease from day 5 to day 8, but not the absolute level of disease, was more dramatic on 2 week old plants, and became less and less so on plants of 4, 6 and 8 weeks of age. In contrast, on the more resistant variety Eston, the rate of increase in disease was similar on plants of all ages, but the amount of disease at each rating date was higher the older the plants were.

Fig 4.1. Development of stemphylium blight on 2, 4, 6 and 8 week old lentil plants of CDC Milestone and Eston. (Y-bars represent are standard errors of the mean)



General Discussion:

Results of these experiments confirm field observation showing that stemphylium blight is more prominent late in the growing season when plants have become more susceptible, and when a thick canopy induces a conducive microclimate. For breeding purposes, the data suggests that in order to differentiate between susceptible (e.g. CDC Milestone) and resistance (e.g. Eston)

genotypes, 2 to 4 week old plants should be inoculated because at that growth stage differences between Eston and CDC Milestone were most pronounced.

5. The Effect of temperature on germination and disease development

Several experiments were conducted using glass slides to determine various factors that influence germination of conidiospores of *S. botryosum*. The use of glass slides rather than whole plants allow these experiments to be conducted in a more efficient way and are used to determine the parameters for whole plant experiments.

5.1 The effect of temperature on germination

Materials and Methods:

The percentage germination of conidia of isolates SB-19 and SB-9 was studied at 5, 10, 15, 20, 25 and 30°C. Spores were harvested from 7-day old cultures grown on V8/PDA medium as described above, and spore concentration was adjusted to $5x10^4$ spores mL^{-1}. Aliquots of 100µl of spore suspension were spread on well slides and incubated at high humidity in a Petri dish with moistened filter paper. Percentage germination was first assessed at 2 hours after the inoculum was placed on the glass well slide. One plate was removed at each assessment time and percentage germination was estimated by examining 100 conidia within several fields of view selected from left to right across each slide. Spores were considered to have germinated when the germ tube was at least half the width of the spore. The number of germinated spores was expressed as a percentage of the total number of spores counted.
Data was tested for homogeneity of variance and normal distribution, and was transformed wherever necessary. Analysis of variances was conducted using either the GLM procedure or the MIXED procedure in SAS. Means were either separated by LSD or Tukey statistics.
Comparisons between treatments using contrasts were made wherever appropriate.
A response surface for the percentage conidia germination as a function of temperature and wetness duration was computed. The response surface regression for each isolate was analyzed using the two-step GLM procedure. The best least squares regression equation predicting conidial germination of *S. botryosum* as influenced by temperature and wetness duration was selected. The selection was based on the coefficient of determination, intercept and the scatter plots.

Results:

Incubation temperature had a significant effect on germination and both isolates had similar germination curves (Fig.5.1 and Table 5.1). During the period of 2 to 6 h of incubation, germination rates at temperatures of 10 to 25°C were very similar, that at 30°C significantly higher, and at 5°C significantly lower. Differences in germination became more pronounced after 8h of incubation and generally, the higher the temperature, the more germination was observed at each point in time. At a temperature as low as 10°C, approximately 10% of conidiospores had germinated after 2 h. The increase of germination at this temperature was lower compared to

germination at higher temperatures, but reached 30% after 20 h of incubation. This suggests that even during cool and wet weather periods infection with *S. botryosum* is possible.

Fig. 5.1. Percent of germination of conidiospores of *Stemphylium botryosum* isolates SB-9 and SB-19 incubated at 5, 10, 15, 20, 25 and 30°C on glass slides over a period of 20h. (Y-bars are standard errors of the means)

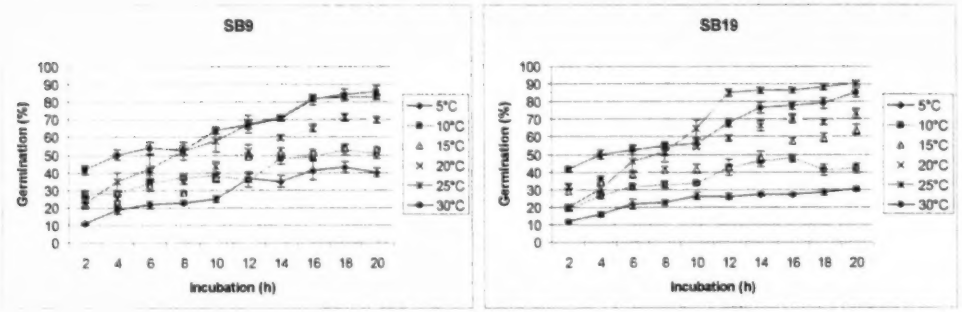


Table 5.1: Percent germination of condiospores of isolates SB-9 and SB-19 after 12 h incubation on glass slides at 5, 10, 15, 20, 25 and 30°C.

Temperature (°C)	SB9 (%)		SB19 (%)	
5	29	e	24	e
10	40	d	36	d
15	40	d	46	c
20	49	c	56	b
25	60	b	65	a
30	65	a	64	a

Means with same letter are not significantly different at p=0.05

The response of conidial germination (*pcg*) to temperature (t) and wetness duration (w) was described by least squares regression equation for SB 9 (Equation 2) and SB 19 (Equation 3).

$$pcg = 7.855 + 0.289(t) + 0.013(t)^2 + 2.167(w) + -0.039(w)^2 + 0.061(t)(w) \qquad r^2 = 0.91 \quad (Eq.\ 2)$$

$$pcg = -5.301 + 2.438(t) + -0.051(t)^2 + 2.182(w) + -0.076(w)^2 + 0.091(t)(w) \qquad r^2 = 0.94 \quad (Eq.\ 3)$$

5.2 Effect of temperature on disease development

Materials and Methods:

Lentil cultivar CDC Milestone was grown in 10 cm diameter plastic pots at 4 plants per pot in a growth chamber at 25°C with a photoperiod of 16 h light/day provided by fluorescent lamps with light intensities of between 315 and 325 $\mu molm^{-2}s^{-1}$. The plants were fertilized with complete

fertilizer solution (20:20:20 NPK + micronutrients) at two weeks after planting at a rate of 3 g per litre and 22 ml per pot.

Petri plates of isolates SB-19 were incubated at 27°C under cool fluorescent light (44 μmol m^{-2} s^{-1}) and photoperiod of 16 h light/day. The conidia were collected from the Petri dishes by flooding the plates with deionized water and then gently dislodging the conidia with a soft brush. A drop of the surfactant and spreading agent, Tween 20 was added to every 1000 ml of deionized water.

Twenty one day old plants were placed in polythene plastic sheets and then inoculated until runoff with the conidial suspension ($2x10^5$ conidia mL^{-1}) using a CO_2 air brush set at pressure of 138 kPa. The inoculated plants were placed in a mist chamber with a humidifier at 10, 15, 20, 25 and 30°C to expose them to a range of leaf wetness periods. Deionized water solution was sprayed on non-inoculated checks. The plants were left in the mist chamber for 0h, 2h, 4h, 6h, 8h, 10h, 12h, 24h and 48h. The plants were allowed to dry in the same growth chamber outside the misting tent under cool fluorescent light with light intensities of 315 μmol m^{-2} s^{-1}. Disease severity was recorded at 3, 7 and 14 days using the HB scale. The area under the disease progress curve (AUDPC) was calculated for ratings at 3, 7 and 14 days after inoculation.

$$AUDPC = \Sigma_i^{n-1}(y_i + y_{i+1}/2)(t_{i+1} - t_i) \qquad (Eq.\ 1)$$

where y_i is the percent severity observed for the i^{th} treatment, t_i is the date of the observation and observations were made on n dates.

Non-parametric tests were used to analysis the data because of its nonconformance to equal variance although the residuals approached normality. The PROC MIXED procedure was conducted on the ranked means from the split plot design with temperature as the main plot and temperature levels were compared by means of contrasts. The LD_CI macro by Brunner was used to estimate the relative effects as well as their standard errors and confidence intervals. The significance of the temperature x wetness duration was determined by the F1_LD_F1 macro.

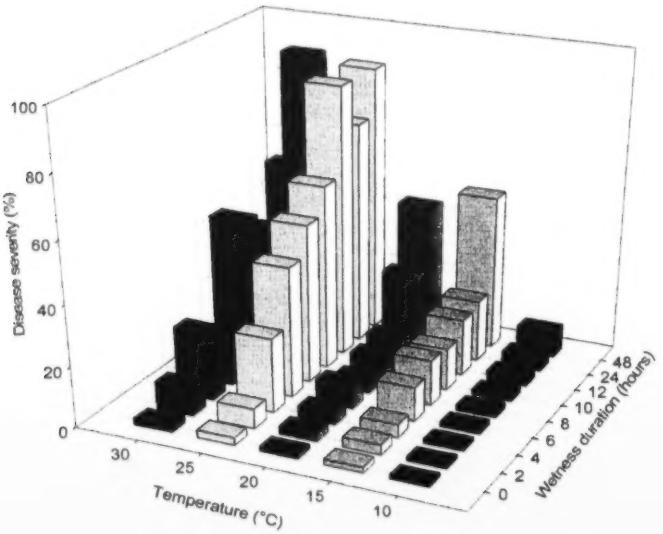
A regression analysis was conducted to determine the form of the relationship of disease severity after 14 days from inoculation with temperature and wetness duration using the Generalized Additive Model procedure and the Generalized Linear Model procedure in SAS. Goodness of fit was evaluated for the models by determining whether the scaled deviance divided by the degrees of freedom is less than 1. The plot of the predicted residuals against observed severity residuals were also used in assessing model fit.

Results:

Stemphylium blight was not observed on the uninoculated plants but the disease was prevalent on all inoculated plants. There was a significant increase in disease severity with increases in the duration of leaf wetness at temperatures of 10, 15, 20, 20, 25 and 30°C (Fig 5.2). The maximum disease severity was observed at 25 and 30°C with leaf wetness periods of 48 h. A rapid increase in disease severity with increasing in leaf wetness duration was found at 25°C although there were no significant difference in disease severity between 25 and 30°C after 48 h of leaf wetness. Disease progressed significantly faster at temperatures of 25 and 30°C compared to lower temperatures, i.e. the fungus required significantly longer wetness periods at lower temperatures to cause as much disease as observed after a few hours of leaf wetness at 25 and 30°C. For

example, at 20°C, 48h of incubation were required to achieve a disease severity level of approximately 40%, whereas the same level of disease was observed after 6h at 30°C (Fig. 5.2).

Fig 5.2. Average stemphylium blight severity on lentil inoculated with a conidial suspension of *Stemphylium botryosum* and incubated over a range of temperatures and leaf wetness duration.



The estimated relative marginal effects ranged from 0.03 to 0.95, with most of the higher values being observed at longer leaf wetness duration and higher temperatures (data not shown). Comparison of means for different temperature levels revealed that there were significant differences among the levels of the two parameters. The effect of leaf wetness duration on disease severity was significantly different at all levels except for 8 h and 10 h.

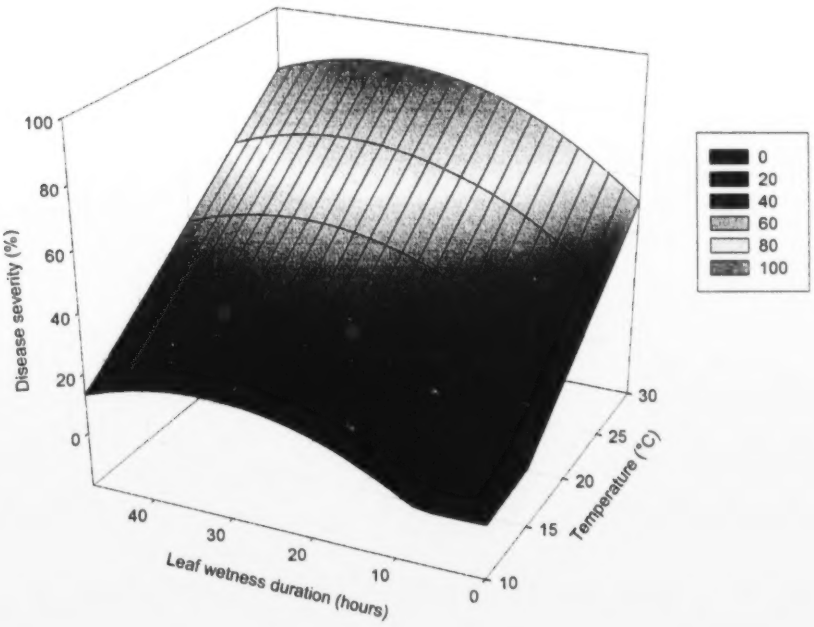
A regression model was developed that predicts the form of dependence of disease severity (*ds*) on temperature (*t*) and leaf wetness duration (*w*). Linear terms for temperature and leaf wetness duration were highly significant in the analysis ($P < 0.001$). The linear and quadratic terms were described in the GENMOD model and the results were consistent with the PROC GAM procedure. The contrasts tests between the linear and quadratic terms of either temperature or leaf wetness duration were highly significant (data not shown). The following model described the relationship between disease severity, temperature and leaf wetness duration:

$$ds = -47.101 + 3.277\ (t) + 2.156\ (w) + -0.033\ (w)^2$$

The scaled deviance divided by the freedom is almost equal to 1 and the R^2 of predicted against observed disease severity ranged from 0.77 to 0.92. The model accurately predicted the response at 10°C where $R^2 = 0.92$. The model overestimated the disease severity in most cases, compared to the observed values. However, the model is still useful in predicting the development of

stemphylium in response to temperature and wetness periods. A response surface was generated based on the regression for 48h of leaf wetness duration and a temperature range of 10 to 30°C (Fig. 5.3).

Fig 5.3. Response surfaces predicting the disease severity of stemphylium blight over a range of leaf wetness durations (2 h - 48 h) at 10, 15, 20, 25 and 30°C. The surface response was generated based on the regression model $ds = -47.101 + 3.277\ (t) + 2.156\ (w) + -0.033\ (w)^2$.



General Discussion:

Experiments using spore suspensions on glass slides as well as inoculated plants and exposing them to a range of wetness periods at different temperatures revealed a significant interaction between temperature and wetness periods. Longer wetness periods were required at lower temperatures for spore germination and for achieving higher disease severity. Highest germination rates and highest disease severity on CDC Milestone were observed at 25 to 30°C. However, even at 5°C approximately 25% of conidia had germinated after 12 h of incubation in wet conditions, and low levels of disease developed at temperatures of 10°C. This observation was very important as rain events in the Prairies even in summer are usually associated with lower temperatures. Some lentil fields were identified with considerable stemphylium blight infection in August 2004. That month was characterized by several periods of cloudy days with significant precipitation and prolonged hours (20 to more than 40h) of high humidity at moderate average temperatures of 10 to 13°C, which would have been sufficient for a slow but consistent increase in infection based on our data.

6. Effects of different dry and wet periods on germination and disease development

Weather patterns during the growing season in Saskatchewan are usually characterized by more or less frequent, shorter rain events, often in the form of thunderstorms, followed by dry periods that in turn may be followed by another cycle of rain events. Fungal spores are therefore exposed to repeated wet and dry cycles that may influence their capability to remain viable. Experiments were conducted to investigate the effects of variable wet and dry cycles on the germination of spores and disease development.

6.1 The effect of intermittent dry periods on germination

Materials and Methods:

Isolate SB-19 was grown as described above. Aliquots of 100µl of spore suspension at $5x10^4$ spores mL^{-1} were spread on well slides and incubated at high humidity in Petri plates with moistened filter paper. Petri plates were incubated at 25°C as follows to determine whether the length of an intermittent dry period had an effect on germination:

Treatment 1: 24 h wetness (control)
Treatment 2: 8 h wetness (control)
Treatment 3: 2 h wetness – 12h dry – 6h wetness
Treatment 4: 2 h wetness – 24h dry – 6h wetness
Treatment 5: 2 h wetness – 48h dry – 6h wetness
Treatment 6: 2 h wetness – 72h dry – 6h wetness
Treatment 7: 2 h wetness – 96h dry – 6h wetness

In a second experiment, spores were exposed to the following incubation conditions to determine whether the length of wetness period before a dry period had an effect on germination:

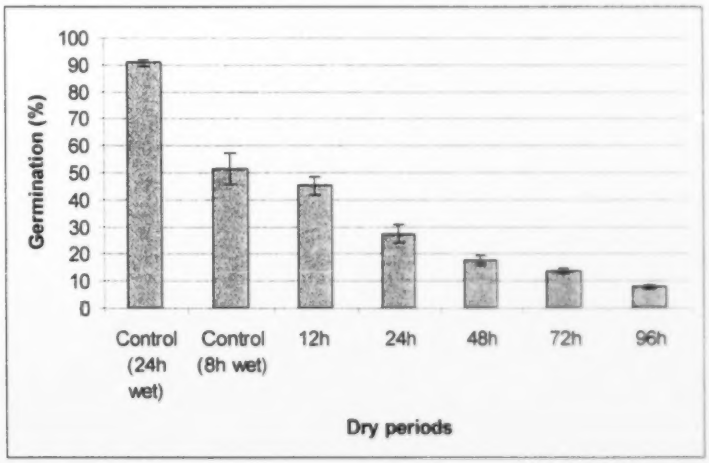
Treatment 1: 24 h wetness
Treatment 2: 18 h wetness
Treatment 3: 12 h wetness
Treatment 4: 2 h wetness – 12 h dryness – 10 h wetness (12 h total wetness)
Treatment 5: 4 h wetness – 12 h dryness – 8 h wetness (12 h total wetness)
Treatment 6: 6 h wetness – 12 h dryness – 6 h wetness (12 h total wetness)
Treatment 7: 2 h wetness – 6 h dryness – 16 h wetness (18 h total wetness)
Treatment 8: 4 h wetness – 6 h dryness – 14 h wetness (18 h total wetness)
Treatment 9: 6h wetness – 6 h dryness – 12 h wetness (18 h total wetness)
Treatment 10: 12h wetness – 6 h dryness – 6 h wetness (24 h total wetness)

Results:

The experiment was designed to imitate possible conditions in the field where spores were imagined to be exposed to a short rain shower (2h wet incubation), and then more or less

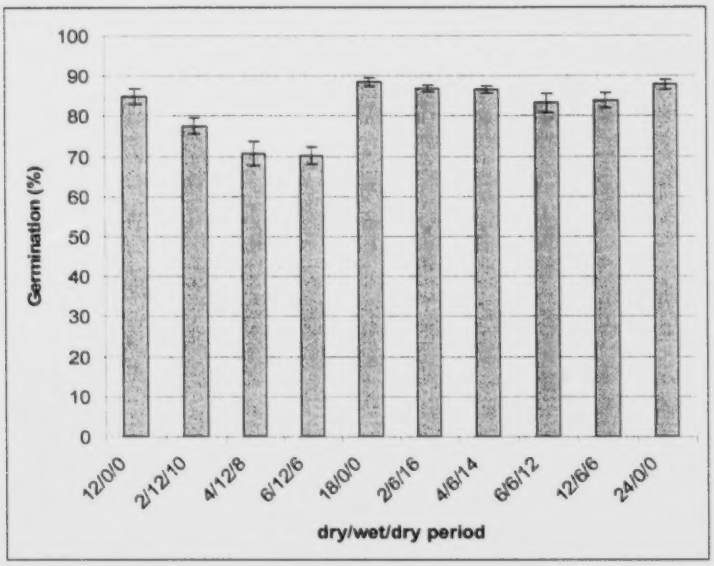
extended dry periods, before they were rewetted by another rain shower. The length of the dry period had a significant effect on the germination rate of conidiospores (Fig. 6.1). Germination rate for 24h under wet conditions (control) reached 90%, but declined to 51% when spores were exposed to 8h wetness only. The germination rate after 8h of incubation was not significantly different from that where spores had been exposed to a 12h dry period, but declined significantly when the dry period was extended beyond 12 h. After a dry period of 4 days the germination rate was below 10% suggesting that most of the spores had been killed. The results suggest that conidiospores of *S. botryosum* are relatively sensitive to dry periods beyond 12h.

Fig. 6.1. Percent of germination of conidiospores of *Stemphylium botryosum* isolate SB-19 incubated for 2h in wet condition, followed by dry periods of 12, 24, 48, 72 and 96h, and incubated for another 6 h under wet conditions. Y-bars are standard errors of the means.



In a second experiment the effect of various wetness periods before a dry period was evaluated, for treatments with a total of 12 and 18h wetness. The initial wetness period allows the spore to start swelling, and, depending on the length of this period, to initiate germination. During this period a spore may vary in its sensitivity. For example, if it initiated germination, it may be more sensitive than if it has not reached that stage yet. Generally, more spores germinated on slides that were exposed to 18h of total wetness compared to only 12h (Fig. 6.2). A period of 12h continuous wetness resulted in significantly higher germination compared to treatments where 12h wetness was interrupted by a dry period. Interrupted dry periods in treatments with 18h total wetness, however, did not result in significantly lower germination compared to 18h continuous wetness. An increase in the length of the wet period before the dry period appeared to reduce germination on slides exposed to 12h and 18h of wetness in total. However, the differences were not significant. This suggests that the duration of the pre-germination wetness period is not significant, but that the total length of wetness periods influences germination.

Fig. 6.2. Percent of germination of conidiospores of *Stemphylium botryosum* isolate SB-19 incubated for various combinations of wet and dry periods. Y-bars are standard errors of the means.



6.2 Effect of intermittent dry periods on disease development

Materials and Methods:

Whole plants were exposed to either continuous or interrupted leaf wetness after inoculation with conidial suspensions. The plants were grown in the growth chamber and the conditions are described under section 3.2.1. The treatments consisted varying wet-dry-wet durations as follows: 4h-6h-44h, 4h-12h-44h, 4h-24h-44h, 8h-6h-40h, 8h-12h-40h, 8h-24h-40h, 12h-6h-36h, 12h-12h-36h, 12h-24h-36h, 24h-6h-24h, 44h-12h-4h, 24h-24h-4h. Thus all treatments received 48 h of total wetness. Continuous wetness of 48 h served as the control. The effect of interrupted leaf wetness on disease development was tested at 20, 25 and 30°C.
The plants were placed in a mist chamber with two humidifier within a growth chamber for the varying wetness periods immediately after inoculation. The RH of 95% and 80% ± 3% was maintained in the plastic mist tent chamber and within the chamber, respectively. After the first initial wetness period, the plants were allowed to dry on the bench at each respective temperature under fluorescent light (315 µmol m^{-2} s^{-1}) which took almost 15 minutes. The plants were placed back into the mist chamber for the remainder of the second wet period. Disease severity was assessed after 14 days using the HB scale. Pots within each chamber maintained at a certain

temperature were arranged in a randomized complete block design. The experiment was repeated twice.
Data was analysed using the mixed procedure in SAS and replicates for each treatment at each temperature were identified as subsamples. Means were separated by LSD and groups were compared using contrasts.

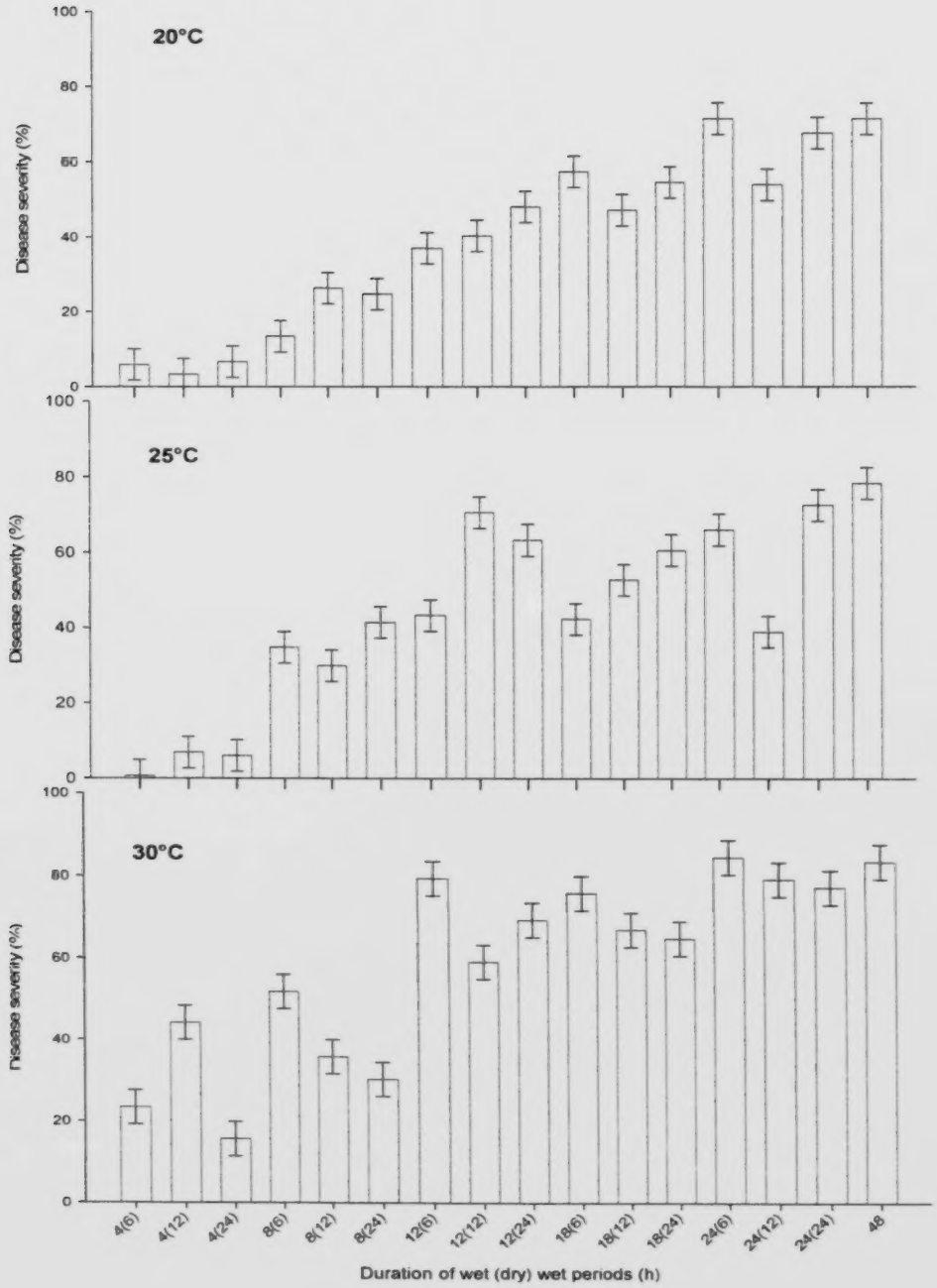
Results:

As previously observed, disease severity was significantly influenced by temperature and higher temperatures resulted in higher disease levels (Fig. 6.3). Plants of all treatments with interrupting dry periods had significantly lower disease levels than those exposed to continuous wetness. However, disease severity on plants exposed to an initial wetness period of 24h only had marginally lower levels of disease compared to the control plants raised incubated in 48h continuous wetness. Each increase in the initial wetness period resulted in a significantly higher disease level on the plants. The effect of the length of the dry period was marginal.

General Discussion:

Results from these experiments demonstrate that the effect of intermittent dry periods depends upon the length of that period, the temperature, the duration of the initial wet period and the period of wetness the spores are exposed to in total. Expanding dry periods beyond 12 h with a total wetness duration of 8 hours results in a significant decrease in spore germination. After an initial wetness period longer than 4 hours spores appeared to be somewhat more susceptible to drying than earlier suggesting that they may reach a critical point in their development that is more sensitive to drying than other stages of development. When air humidity was maintained at 80% during the dry period, the fungus appeared to be less sensitive to dry periods at 30°C compared to 25 or 20°C. This may be due to the fact that its development is faster at this temperature thus dry-sensitive stages in the development are passed through quicker. Increasing initial wetness periods resulted in higher disease severity, suggesting that more spores were successful at invading the longer they were exposed to free water on the leaf surface. This effect was more significant than the duration of the dry period up to 24h.

Fig 6.3. Effect of interrupted wetting periods on infection of lentil by *Stemphylium botryosum* at 20, 25 and 30°C. Each interrupted wetness treatment was exposed to 48 h of wetness in total.



7. Effect of *S. botryosum* on a selection of Canadian lentil cultivars

A wider range of cultivars was subsequently tested with a moderately virulent isolate of *S. botryosum* to further evaluate differences in resistance to the pathogen.

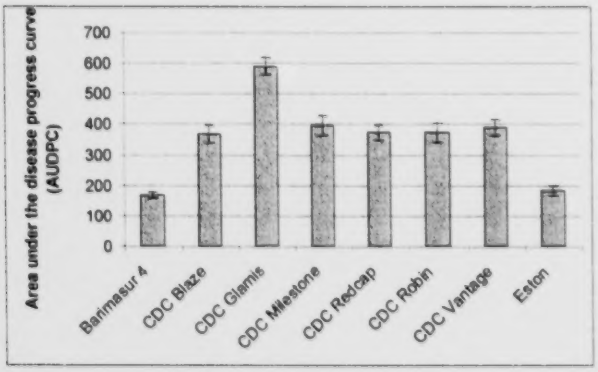
Material and Methods:

Eight lentil cultivars (CDC Blaze, CDC Vantage, CDC Glamis, CDC Milestone, CDC Robin, CDC Redcap, Eston and Barimasur 4) were grown for 2 weeks at 5 plants per pot and 6 replicate pots per cultivar as described before. Plants were inoculated with a spore suspension of SB-19 at 2×10^5 spores mL^{-1} after 2 weeks, incubated in a mist chamber for 48 h and then moved to a greenhouse bench, both times in a completely randomized design. Disease severity was rated 4 times starting 5 days after inoculation and every 4^{th} day thereafter using the Horsfall-Barratt scale. Data was transformed into percentage data and the area under the disease progress curve was calculated for data analysis. Data was transformed by square root transformation and analyzed using the mixed procedure in SAS. Means were separated by LSD.

Results and Discussion:

Final disease ratings 17 days after inoculation ranged from 9% (Barimasur 4) to 95% (CDC Glamis). Barimasur 4 and Eston were the most resistant cultivars with significantly lower disease levels compared to all other lentil cultivars (Fig. 7.1). Glamis was the most susceptible variety with significantly higher levels of stemphylium blight compared to all other lentil cultivars tested. CDC Blaze, CDC Milestone, CDC Redcap, CDC Robin and CDC Vantage had comparable, intermediate levels of resistance.
These results suggest that Barimasur 4 and Eston possess resistance gene(s) to the pathogen. Barimasur 4 is currently used in the breeding program to transfer resistance to adapted varieties. It would be interesting to investigate whether Eston has the same resistance genes or others, in which case combination of bother sources of resistance may result in superior resistance to the pathogen.

Fig. 7.1. Disease severity measured as Area Under the Disease Progress Curve (AUDPC) of *Stemphylium botryosum* inoculated onto 3-week old plants of 8 lentil cultivars.



8. The effect of stemphylium blight on biomass production and yield

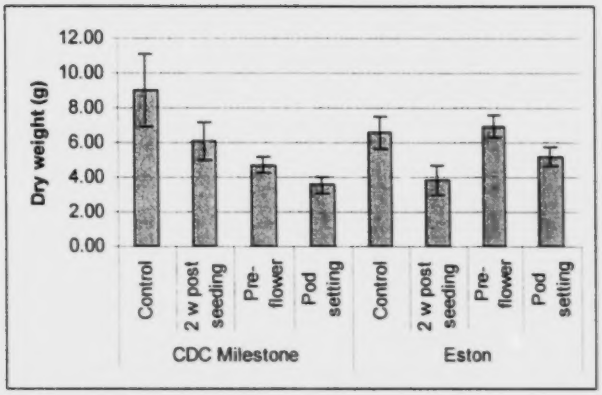
Materials and Methods:

Isolate SB-19 was grown V8/PDA medium and incubated at 27 ^{O}C for 25 days (11 days light/ 3 days dark/11 days light).
CDC Milestone and Eston were planted in 6 replications at a density of 1 seed per one gallon pot and inoculated at 2 weeks after seeding, the pre-flower stage and pod setting stage, with the concentration of $2x10^{5}mL^{-1}$. The spore suspension was applied with an air brush and a carbon dioxide applicator at 5 mL of spore suspension per plant for plants inoculated 2 weeks after seeding, and at 10 mL per plant for later inoculation to take account of the larger size of plants at that stage. Following inoculation, plants were placed in a mist chamber for 48 hr. All treatments were kept on a mist bench during the ratings. Disease severity was rated 5 days after inoculation using the Horsfall-Barrett (0-11) scale. The ratings were repeated 5 times every 4 days. After all ratings were recorded, plants were maintained until harvest. The dry weight of plants and the seed weight were determined after harvesting. A standard method was used measuring seed size using sets of mesh screens.
Data was tested for homogeneity of variance and normal distribution, and was transformed wherever necessary. Analysis of variances was conducted using either the GLM procedure or the MIXED procedure in SAS. Means were either separated by LSD or Tukey statistics.
Comparisons between treatments using contrasts were made wherever appropriate.

Results:

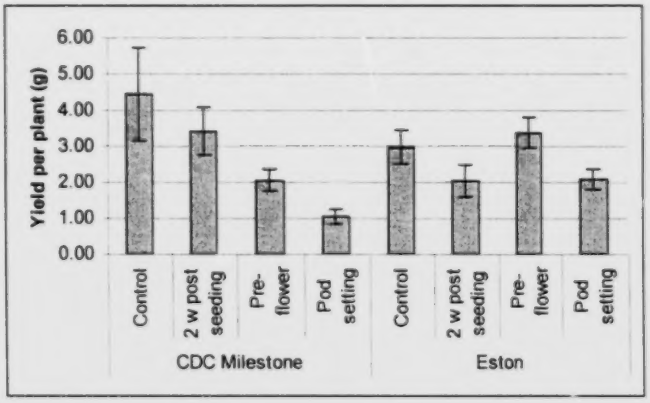
The effect of stemphylium blight on the dry weight of the biomass at harvest varied between CDC Milestone and Eston (Fig.8.1). Control plants of CDC Milestone had a significantly higher dry weight compared to plants inoculated at prep-flower and at pod setting. Plants of Eston inoculated 2 weeks after seeding produced significantly less biomass compared to those inoculated a pre-flower. Control plants, inoculated plants at pre-flower and at pod setting had similar dry weights suggesting that only an early infection affected biomass production in this, more resistant variety.

Fig. 8.1. Dry weight of biomass produced by plants of CDC Milestone and Eston after inoculation with *Stemphylium botryosum* at various growth stages. Y-bars represent are standard errors of the mean.



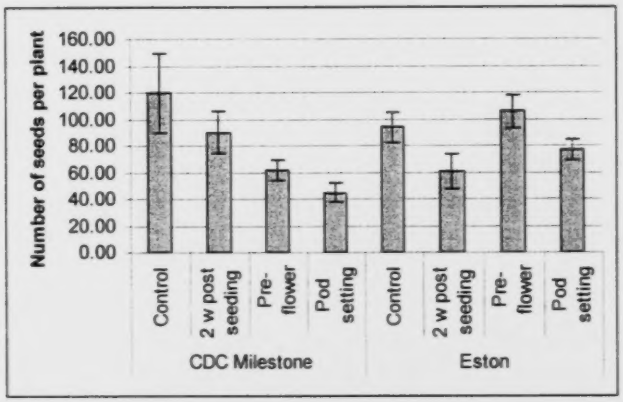
A similar trend was found when analyzing yield data (Fig. 8.2). There was a significant reduction in yield on plants of CDC Milestone inoculated at pre-flower and at pod setting compared to the control. Plants inoculated 2 weeks after seeding had similar seed weights to control plants, but produced more seed than plants inoculated at pod setting. In contrast, differences in yield between treatments in Eston were not significant.

Fig 8.2. Seed yield (g per plant) produced by plants of CDC Milestone and Eston after inoculation with *Stemphylium botryosum* at various growth stages. Y-bars represent are standard errors of the mean.



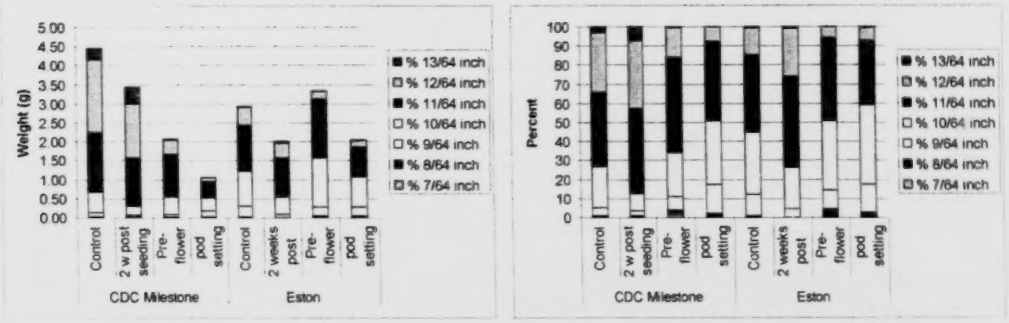
Similar to seed weight, CDC Milestone control plants produced significantly more seeds per plant than those inoculated at pre-flower and at pod setting (Fig 8.3). Plants inoculated 2 weeks after seeding had similar numbers of seeds than control plants, but significantly more than plants inoculated at pod setting. Eston plants inoculated at pre-flower had marginally more seeds compared to plants inoculated 2 weeks after seeding.

Fig. 8.3. Seed yield (number of seeds per plant) produced by plants of CDC Milestone and Eston after inoculation with *Stemphylium botryosum* at various growth stages. Y-bars represent are standard errors of the mean.



Seeds harvested from plants were screened through a set of 7 sieves with decreasing mesh sites to determine seed size fractions. Results are presented in grams and in percentage (Fig. 8.4). Results suggest that inoculations at pre-flower and pod setting result in a decrease of the two largest seed fractions and a simultaneous increase in the 3 smallest fractions. The fraction of 11/64 inch remained relatively constant.

Fig. 8.4. Proportion of size fractions of seed (in g and percentage) produced by plants of CDC Milestone and Eston after inoculation with *Stemphylium botryosum* at various growth stages.



General Discussion:

Results suggest that resistant and susceptible varieties, such as CDC Milestone and Eston show a different response to infection at different stages of their development with respect to biomass and yield. However, further varieties and genotypes have to be tested to confirm these trends. Also, estimates of biomass and yield loss due to disease under controlled conditions are

notorious for not correlating well with plant reaction under field conditions; therefore it would be useful to conduct further studies under field conditions.

9. Seed transmission of *S. botryosum*

Seed from Experiment 8 were supposed to be used to test for the level of seed infection and for the rate of seed-to-seedling transmission of *S. botryosum*. However, the amount of seed available form this experiment was too low to supply seeds for all tests. Therefore, a new set of plants was inoculated and the seed from these plants were further tested.

9.1 Seed infection rates on lentil plants inoculated with *S. botryosum*

Materials and Methods:

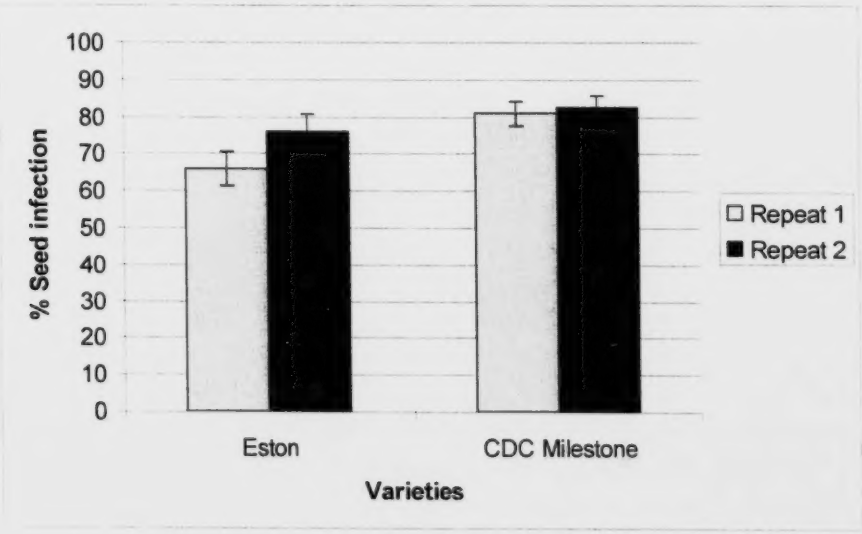
CDC Eston and Milestone were grown in 6 replicate pots each for inoculation as described above. Due to difficulties in producing sufficient spores for large-scale inoculations, plants were inoculated with a mycelial suspension prepared following a standard protocol.
Cultures of a SB-19 were grown on PDA+V8 juice and incubated at 27 ^{O}C for 25 days. Ten plugs from 25 days old culture were subsequently used to inoculate 500 ml sterile modified Richard's medium (10 g sucrose, 10 g KNO3, 5g KH2PO4, 2.5 g MgSO4.7 H2O, 0.02g FeCl3, 150 ml V-8 juice, 850 mL distilled water, pH adjusted to 6.0 with NaOH). The inoculated bottles were plugged with sterilized cotton and incubated on a rotary shaker at 15°C at 110(r/min) for 10 days. Aseptic conditions were maintained during the harvest of mycelia and the reminder of the procedure. Liquid media (500 mL) containing the mycelium were filtered under vacuum for 3 minutes. An aliquot of 500 mL sterile di-ionized water was added to the bottle to wash out all remaining mycelium. Mycelium was separated from the liquid by filtration twice prior to transfer into sterile Petri dish. The lids were left open a small amount and the plates were left in an air flow cabinet for 48 hr. The air-dried mycelium was ground for 1 minute with a coffee grinder then passed through a 0.83mm screen to separate out the big particles to prevent plugging of the air brush nozzle. In preliminary experiments, dilution series had been prepared that were plated onto PDA to determine colony forming units (CFU), parallel to taking readings of these dilutions with a spectrophotometer at 600 nm wavelengths. Dilutions had also been evaluate by plant inoculations to determine a suitable mycelial CFU concentration, demonstrating that 0.5g of mycelium L^{-1} H_2O (approximately 25,438 CFU with a spec reading of approximately 0.422) inoculated onto CDC Milestone resulted in disease levels similar to that of inoculations with 1 x 10^5 spores mL^{-1}.
Plants were inoculated twice with a mycelial suspension of 0.5g mycelium L^{-1} (spectrophotometer reading of 0.4), once at mid podding and the second time at late podding. After inoculation, plants were incubated in a mist chamber for 4 days and were then transferred to greenhouse benches with a misting system where they remained until maturity. Uninoculated control plants of both varieties were included.
Seed was harvested from each replicate pot separately in the first repeat of the experiment, but was bulked for each variety and subsequently randomly divided into 6 subsamples for seed testing in the second repeat.

For each replicate, 20 seeds were randomly picked, surface-sterilized in 10% bleach for 5 minutes and plated at 5 seeds per Petri plate onto tap water agar. Plates were incubated for 7 days at room temperature at which point the number of seeds with stemphylium blight infection was determined. Data was tested for homogeneity of variance, transformed by arcsine transformation, pooled for the two runs and analyzed using the mixed procedure in SAS.

Results and Discussion:

Disease severity on inoculated plants at maturity was high reaching between 8 and 9 on the scale described under 3.2. Seed from uninoculated lentil plants showed no signs of infection by *S. botryosum*. Seed infection levels were somewhat more variable in the first repeat which may have been due to the fact that seeds were plated in replicates based on pots from which they had been harvested, whereas seeds from the second repeat were bulked and then subdivided into subsamples for plating. Seed infection rates were high with levels well above 50% even though lentil plants were only inoculated later during their development at mid and late podding (Fig. 9.1). There was no significant difference in seed infection levels between CDC Milestone with an average of 82% seed infection and Eston with an average infection level of 71%. Highly conducive conditions were maintained after inoculation allowing the fungus to grow rapidly and without interruption, which may explain the high seed infection levels.
Seed infection levels in this test are significantly higher than what has been observed in field samples. This may be due to the fact that infection in the field occurs later in the field than initiated in these experiments under controlled conditions. Significant symptoms of stemphylium blight on leaves in 2004 and 2005 were only observed in mid to late August, which may not leave enough time for the fungus to infect seed until maturation of the crop thereby resulting in lower seed infection rates.

Fig. 9.1. Infection rates of seed from CDC Milestone and Eston inoculated with *Stemphylium botryosum* at mid podding and again at late podding.



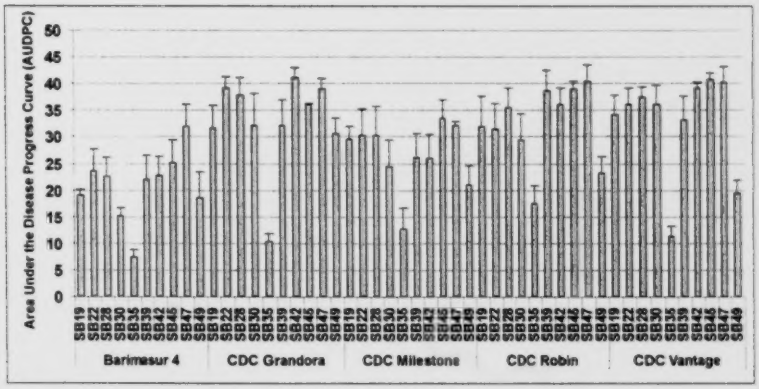
10. Virulence among isolates

Ten isolates of *S. botryosum* were randomly selected and inoculated onto 5 lentil cultivars to investigate whether there are differential interactions between isolates and cultivars.

Materials and Methods:
The following 10 isolates of *S. botryosum* were randomly selected from the collection of isolates obtained from infected lentil seed through Discovery Seed Lab: SB-19, SB-22, SB-28, SB-30, SB-35, SB-39, SB-42, SB-45, SB-47, SB-49. Mycelial suspensions were prepared as described before. Plants of Barismasur 4, CDC Grandora, CDC Milestone, CDC Robin and CDC Vantage were grown for 3 weeks at 4 plants per pot and 4 replicate pots per isolate-cultivar combination as described previously. The experimental design was a RCBD with blocks arranged over time. Plants were inoculated with a mycelial suspension prepared as described previously and diluted to obtain a spectrophotometer reading of 0.4 at 600 nm. Plants were incubated in a mist chamber for 72h and pots were then moved to a greenhouse bench where they were rated 4, 7 and 11 days after inoculation. Disease data were summarized as area under the disease progress curve (AUDPC), transformed by square root transformation and analyzed using the GLM procedure in SAS. Means were separated using Tukey's Honestly Significant Difference (HSD).

Results and Discussion:
Isolates and cultivars had a highly significant effect on disease severity, whereas the interaction of cultivar x isolates was not significant, suggesting that there is no differential interaction between isolates and cultivars. Barimasur 4 proved to be most resistant cultivar with lowest disease ratings for all isolates compared to the other cultivars (Fig. 10.1). CDC Milestone had an intermediate reaction whereas CDC Grandora, CDC Robin and CDC Vantage were equally susceptible to isolates of the pathogen. Variability was also observed among isolates. SB-35 was consistently the least virulent isolate causing significantly less disease than all other isolates. Overall, isolates represented a continuum of increasing virulence without separating into distinct groups. The results suggest that there is no race structure present in the population of *S. botryosum*.

Fig. 10.1. Disease severity measured as Area Under the Disease Progress Curve (AUDPC) of 10 isolates of *Stemphylium botryosum* inoculated onto 3-week old plants of 5 lentil cultivars.



11. Efficacy of fungicides against *S. botryosum*

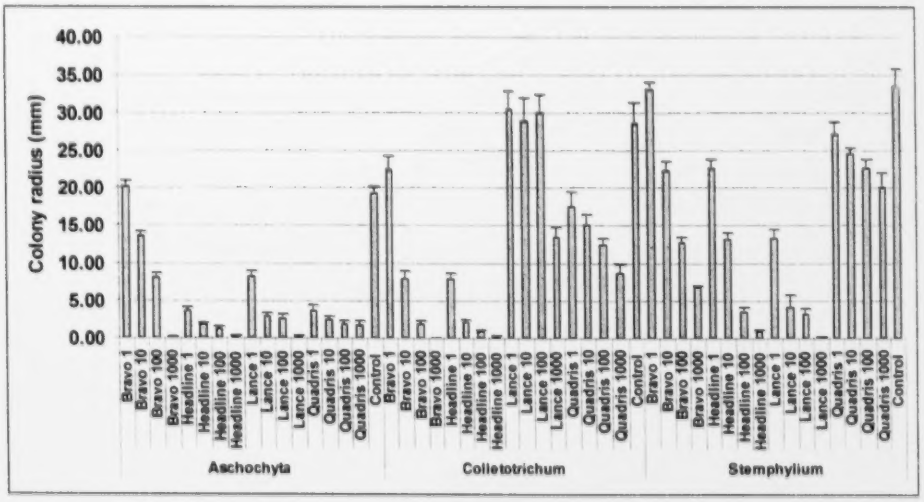
Two experiments were conducted to evaluate the efficacy of fungicides against *S. botryosum*, a plate test using fungicide amended media to investigate inhibition of fungal growth, and screening fungicides on inoculated plants under controlled conditions.

11.1 Effect of fungicides on colony growth of *S. botryosum*

Materials and Methods:
V8/PDA media was prepared as described previously, and was amended with 1000 ppm, 100 ppm, 10 ppm or 1 ppm of Headline, Bravo, Quadris or Lance. Isolates of *Ascochyta lentis*, *Colletotrichum truncatum* and *Stemphylium botryosum* were inoculated onto 4 replicate plates of each fungicide/concentration combination plus 4 control plates without fungicides. Plates were incubated in an incubator at 25°C for 12 days. Colony radius was measured three times per plate and the average radius was used for data analysis.

Results:
The efficacy of fungicide in inhibiting the growth was influenced by the doses and the fungal pathogen. Overall, Bravo was least effective in inhibiting all pathogens. Headline was effective against *A. lentis* and *C. truncatum*, and intermediate against *S. botryosum.* Lance was most effective against *S. botryosum*, intermediate against *A. lentis*, but showed no inhibition except for the highest concentration against *C. truncatum*. Quadris was effective against *A. lentis*, intermediate against *C. truncatum*, and was not very effective against growth of *S. botryosum*. For most fungicides and pathogen, an increase in the concentration significantly decreased growth of the pathogen.

Fig. 11.1. Radial growth of *Ascochyta lentis*, *Colletotrichum truncatum* and *Stemphylium botryosum* on V8/PDA plates amended with different rates (ppm) of Bravo, Headline, Lance and Quadris.



11.2 Efficacy of fungicides in controlling stemphylium blight on inoculated lentil plants

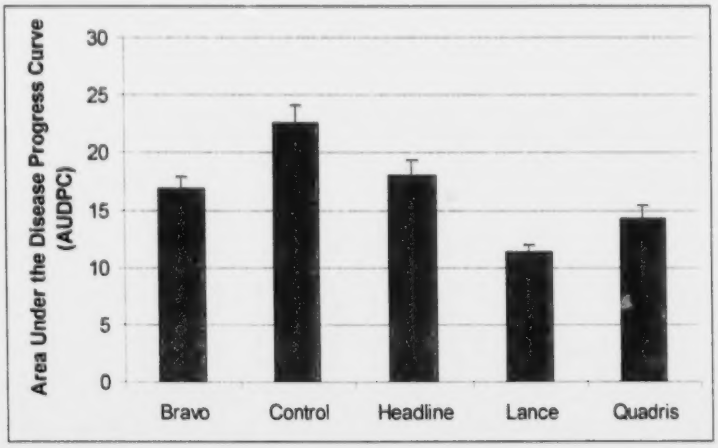
Materials and Methods:

CDC Milestone plants were grown as previously described with 5 plants per pot and 10 replicate pots per fungicide treatment. Plants were grown for 2 weeks and then sprayed with Bravo, Headline, Lance or Quadris at the recommended rate. Inoculated with a mycelial suspension of *S. botryosum* as previously described was conducted 24 h after fungicide application. 10 control plots without fungicide application were also inoculated. Plants were subsequently incubated for 3 (repeat 1) or 4 (repeat 2) days in a mist chamber before being moved to a greenhouse bench. Plants were rated 3, 6, 9 16 days (repeat 1) and 5, 7, 12, 15 days after inoculation. Data was summarized as area under the disease progress curve, square root transformed and analyzed using the mixed procedure in SAS. Means were separated by the LSD procedure.

Results:

Lance and Quadris reduced stemphylium blight significantly compared to the control (Fig.11.2). The effect of Bravo on stemphylium blight was marginally non-significant compared to the control ($P = 0.047$), and Headline treated plants showed no significant reduction in disease severity ($P = 0.078$).

Fig. 11.2. Severity of stemphylium blight measured as Area Under the Disease Progress Curve (AUDPC) on CDC Milestone treated with standard rates of Bravo, Headline, Lance and Quadris.



General Discussion:

Among the fungicides tested Lance appears to have highest efficacy in controlling *S. botryosum* on lentil. It showed highest inhibition of fungal growth in plate tests, best control of the pathogen on inoculated lentil plants. As lentil plants were inoculated with mycelial suspensions, this test

and the plate test evaluated the efficacy of fungicides in inhibiting mycelial growth rather than inhibiting spores. It may be possible that the strobilurin fungicides Quadris and Headline would have shown higher efficacy in controlling stemphylium blight if spore suspensions had been used for inoculation as this group of fungicides is known to be particularly effective in arresting spore germination. However, Lance has a similar mode of action to the strobilurins in disrupting mitochondrial activity in the fungus, though it targets a different site in those organelles. It is known to have a faster systemic activity that may give it a comparative advantage over strobilurins in fast growing pathogens.

Papers/Presentations:

Poster presentation:

Hashemi, P., Vandenberg, A. and S. Banniza, 2005: Developing a protocol for large-scale inoculation of lentil germplasm with *Stemphylium botryosum*. Plant Canada 2005, Edmonton, Alberta, June 15-18, 2005.

Kumar, P., Mwakutuya, E., Hashemi, P., Banniza, S., and A. Vandenberg, 2004. Stemphylium blight of lentil: Should YOU Be Worried? Pulse Days 2004, January 12- 13, Saskatoon, SK. (poster abstract).

Mwakutuya, E., Vandenberg, A., and S. Banniza: Effect of culture age, temperature, incubation time and light regimes on conidial germination of *Stemphylium botryosum* on lentil *(Lens culinaris)*. 5th Canadian Pulse Research Workshop, November 29-30, 2004, London, Ontario. (poster abstract; 1st Prize of student poster presentations)

Mwakutuya, E., Vandenberg, A., and S. Banniza, 2005: Is *Stemphylium botryosum* adapted to the climatic conditions of Saskatchewan? Pulse Days 2005. Saskatoon, January 10-12, 2005 (Poster abstract).

Hashemi, P., Vandenberg, A., and S. Banniza, 2005: Developing protocols for the production of conidiospores of *Stemphylium botryosum* and the inoculation of lentil for resistance breeding. Pulse Days 2005. Saskatoon, January 10-12, 2005 (Poster abstract).

Kumar, P., Mwakutuya, E., Hashemi, P., Banniza, S., and A. Vandenberg, 2004. Stemphylium blight of lentil: Should YOU Be Worried? Pulse Days 2004, Saskatoon, January 12-13 (poster abstract).

Oral presentation:

Mwakutuya, E., Vandenberg, A., and S. Banniza, 2005: Influence of leaf wetness duration, interrupted wetness periods and temperature on the development of stemphylium blight on lentil. Presentation at the Meeting of the Saskatchewan Section of the Canadian Phytopathological Society, December 5th, 2005.

Mwakutuya, E., Vandenberg, A., and S. Banniza, 2005: Etiology of stemphylium blight of lentil (*Lens culinaris*) in Saskatchewan. Plant Canada 2005, Edmonton, Alberta, June 15-18, 2005.

Kumar, P., Banniza, S. and A. Vandenberg, 2004: Stemphylium blight of lentil: Finding sources of resistance. 5th Canadian Pulse Research Workshop, November 29-30, 2004, London, Ontario.

Research reports:

Banniza, S., and A. Vandenberg, 2005: Progress Report: Investigation into the biology of *Stemphylium botryosum*, a potentially new pathogen on lentil production in Saskatchewan. Submitted to SPG, March 2005, pp.21.

Banniza, S., and A. Vandenberg, 2005: Progress Report: Investigation into the biology of *Stemphylium botryosum*, a potentially new pathogen on lentil production in Saskatchewan. Submitted to ADF, December 2004, pp.21.

Banniza, S., and A. Vandenberg, 2003: Investigation into the biology of *Stemphylium botryosum*, a potentially new pathogen in lentil production in Saskatchewan. Annual Reports, Saskatchewan Pulse Growers and Saskatchewan Agricultural Development Fund, 9pp.

Future Research:

In the remaining 3 months of this project experiments will be conducted to assess the seed-to-seedling transmission of *S. botryosum*. We have also been in discussion with BASF about field experiment to study the effect of fungicide on stemphylium blight under field conditions.

Information of benefit to producers, processors, or governments:

Research results from this project show that *S. botryosum* can significantly affect yield and seed quality if infection occurs early enough. Field observations so far suggest that this is not the case and lower seed infection rates in field samples compared to samples from experiments under controlled conditions confirm this. Our results also show that lentil plants are more susceptible the older they are, which is supported by field observations showing that the disease appears to show up late in the growing season. The fungus is active at temperatures as low as 5°C, but requires longer rainy periods to cause any significant damage. This suggests that it may only be a problem in years with more frequent and longer periods of precipitation later in the season. Fungicide trials revealed that Lance appears to be best at controlling the fungus, followed by Quadris. Lance is the recommended fungicide to control other fast growing fungi such as *Botrytis cinerea* and *Sclerotinia sclerotiorum*. Both fungicides are used by producers to control these and other lentil diseases (anthracnose and ascochyta blight). Field trials, which we hope to conduct over the next years, will reveals to what degree stemphylium blight is present in particular in southern Saskatchewan.

(d) **Personnel:**

Edmore Mwakutuya has conducted his MSc program on this project. Pramod Kumar, a MSc student financed through and working under supervision of Dr A. Vandenberg has conducted some experiments in this project that related to his research program (Identifying resistance genes for stemphylium blight). Other experiments were conducted by technician and summer students on an in-kind basis.

(e) Equipment:

None

(f) Project Developed Materials:

A new disease rating scale specifically for stemphylium blight has been developed.

(g) Project Photos:

Project pictures are supplied on CD

(h) Acknowledgement:

The Saskatchewan Agricultural Development Fund and the Saskatchewan Pulse Growers who have co-funded this research project have been acknowledged in all presentations where this research project was mentioned.

(i)Expense Statement:

Will be forwarded